Accuracy of age estimates for larval *Sebastes jordani*

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Abstract.—We examine some issues concerning the accuracy of larval age estimates for *Sebastes jordani*. To localize a starting point for daily increment counts, gestating and planktonic larvae were examined to determine whether an extrusion check forms at parturition. We also assessed 1) the resolution limitations of optical microscopy in ageing larvae, 2) the precision of age estimates, and 3) the effect of specimen age on the reliability of ages.

Preextrusion increments are commonly observed in gestating and planktonic larvae, but a distinctive extrusion check forms in the otolith at parturition. This feature occurs predictably at a radius of 15–19 μm and provides an unambiguous starting point for increment counts. Observations of larval otoliths with a scanning electron microscope revealed that the minimum size of postextrusion increments is ~0.6 μm, which is large enough to be resolved with a high-quality optical system. Cross-checking of ages among three readers showed 84% agreement to within ±1 d and no systematic differences among readers. The clarity of otolith microstructure resulted in high confidence scores for age estimates; however, very young larvae (0–3 d) were the most difficult to age. An exponential growth model fitted to 2,203 aged larvae indicated that during the first month of life, growth in length proceeds at the rate of 2.75%·d^{-1}.

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Ichthyoplankton surveys are used routinely to estimate spawning stock biomass (Gunderson, 1993; Hunter et al., 1993). Fundamentally, this type of calculation requires a determination of total daily egg production (P_0 [eggs·d^{-1}]), which is estimated by regressing egg abundance on egg age. Typically, egg ages are obtained indirectly by 1) description of egg-stage ontogeny, 2) laboratory studies of the temperature dependency of egg developmental rates, 3) measurement of thermal conditions in the environment at the time of sampling, and 4) back calculation of egg age. Not surprisingly, serious complications can occur with this approach, especially when spawning occurs in deep water (e.g. Lo et al., 1993; Picquelle and Megrey, 1993).

Members of the rockfish genus *Sebastes* (family Scorpaenidae) are viviparous, bearing advanced yolk-sac larvae at the time of parturition (Wourms, 1991; Bowers, 1992). Previous work has shown that *Sebastes* larvae can be aged directly by enumeration of daily otolith increments (Penney and Evans, 1985; Laidig et al., 1991). Thus, rockfishes exhibit a life history adaptation, i.e. viviparity, which potentially lends itself to accurate estimation of daily larval production rates (P_0 [larvae·d^{-1}]).

The purpose of this study is to examine the accuracy of larval *Sebastes* age estimates obtained through the study of otolith microstructure (Stevenson and Campana, 1992). To be considered accurate, a sample statistic must be close to the true underlying "population" value it estimates. Inaccuracy is due to the combined effects of estimator bias and imprecision. Although direct ageing of rockfish larvae circumvents many of the problems associated with staging eggs (see above), accurate larval age estimates, characterized by low bias and high precision, are still prerequisite to an accurate larval production estimate of spawning stock biomass. Because larval mortality rates in the marine environment are high, commonly exceeding 0.15·d^{-1} (Pepin, 1991; Houde, 1994), systematic larval ageing errors as small as one day can significantly bias estimates of spawning biomass.

Sebastes jordani is an abundant but lightly utilized rockfish that occurs along the central California coast (Lenarz, 1980). Importantly, it can be readily identified at all early life history stages (Moser et al., 1977). In addition, a detailed growth model for the first six months of life has been previously described (Laidig et al., 1991). Those authors validated a 1:1 correspondence between changes in counts of daily growth increments and age for a developing cohort of pelagic juvenile *S. jordani* (age 3–5 mo).

In this study we examine more closely the accuracy of *S. jordani* age estimates derived from the first month of life. In particular, we address the following questions: 1) Are daily increments large enough to be resolved with an optical microscope (Neilson, 1992)? 2) Does the *Sebastes* "extrusion check" (Penney and Evans, 1985; Laidig et al., 1991) actually form at parturition? 3) How variable are age estimates obtained from different readers? 4) How variable are individual larvae in their expression of otolith microstructure? and 5) Does larval age influence the reliability of age estimates?

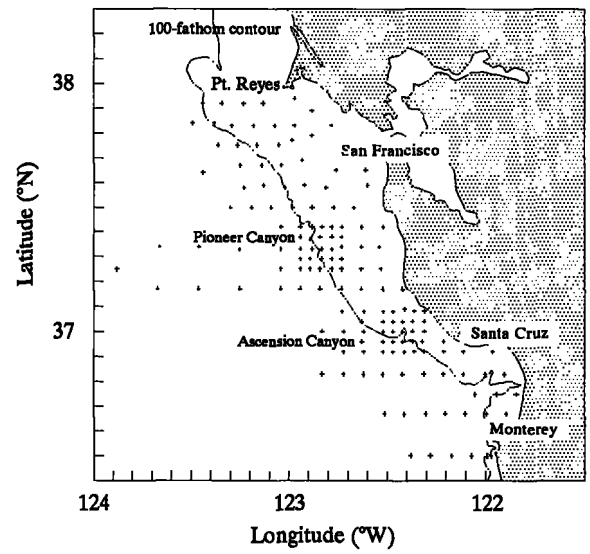

Figure 1

Map of the study region showing the locations of bongo-net stations.

Materials and methods

The primary set of ichthyoplankton samples used in this study was obtained during a cruise aboard the NOAA RV *David Starr Jordan* conducted from 8–15 February 1991. During that six-and-a-half-day period, 150 bongo-net stations were occupied in the region bounded by lat. 36°30'N and 38°00'N, and offshore by a maximum distance of 130 km (Fig. 1). This region is known to harbor large spawning aggregations of *S. jordani* during the months of January and February, especially in the vicinities of Pioneer and Ascension Canyons (Lenarz, 1980; MacGregor, 1986; Chess et al., 1988).

Field and laboratory processing of the samples closely followed prescribed CalCOFI guidelines (i.e. Kramer et al., 1972; Smith and Richardson, 1977). However, because *Sebastes* larvae generally occur in the upper mixed layer (Ahlstrom, 1959; Kenchington, 1991; senior author's unpubl. data), the maximum amount of wire deployed was 200 m, resulting in a maximum depth fished of 140 m. After retrieving and washing down the 505-μm mesh nets, the samples were preserved in 80% ethanol (EtOH), which was changed 24 hours later. In the laboratory the alcohol was changed again, the samples were sorted, and *S. jordani* larvae were identified and enumerated. Sorted larvae from each of the 150 ethanol-preserved bongo nets were randomly subsampled and their otolith microstructure was examined. Notochord length (NL) was measured from each larva in the subsample and the otoliths were extracted and affixed to glass slides with clear fingernail polish.

Individual ages were determined by the methods outlined in Laidig et al. (1991). In brief, otoliths were viewed whole under oil emersion with a compound microscope that was equipped with a video camera and monitor (e.g. McGowan et al., 1987), producing a working magnification of 1,250×. Counts of daily increments were initiated at a distinct check mark that occurred consistently at a radius of ~15–19 μm. The mark clearly encircled the otolith core and also a more recent zone of faint incremental growth. Penney and Evans (1985) and Laidig et al. (1991) both observed this feature and inferred that the check was formed at the time of larval extrusion (i.e. parturition). Otoliths with this mark and no additional increments were therefore given a nominal age of zero. Increments were counted from the mark to the most distal point along the longest growth axis. A digitizer was used to measure the radius (μm) of the otolith at the extrusion check (R_0) and the inner edge of the dark, protein rich D zone of each subsequent daily increment (R_1, R_2, R_3, ..., etc.). During postprocessing the width of each increment (W_i) was then calculated by subtraction (i.e. $W_i = R_i - R_{i-1}$). A series of computer programs automated this procedure (Laidig and Pearson, 1992).

A confidence code ranging from 1–5 was assigned by the reader at the time each larva was aged. The

codes were as follows: 1 = otolith was not readable; 2 = low confidence in age estimate, changing the focal plane may have resulted in a different increment count; 3 = moderate level of confidence, preextrusion increments sometimes evident, extrusion check sometimes at an unusually large or small otolith radius; 4 = high confidence in age estimate; and 5 = otolith exhibits remarkable clarity, no ambiguity concerning increment interpretation. To compare age estimates obtained from different readers and to quantify measurement error, every tenth specimen in a tow subsample was aged independently by two people.

Preextrusion larvae from gestating females were examined to characterize otolith growth prior to parturition. Samples for this study came from another cruise of the RV *David Starr Jordan* (17–27 February 1993). Mature gestating female *S. jordani* were collected by bottom trawl (26-m headrope, 15-cm mesh, 1.25-cm codend), their ovaries were removed, and embryos and prolarvae preserved in 95% ethanol. Prolarvae (i.e. hatched embryos) were staged in the laboratory on the basis of a modified version of Yamada and Kusakari's (1991) system and on the basis of information on the temporal course of development, inferred from laboratory experiments on *Sebastes flavidus* (Eldridge[1]). Specifically, the following prolarval stages were assigned: 1 = parturition in 5–6 d; 2 = parturition in 4–5 d; 3 = parturition in 3–4 d; 4 = parturition in 2–3 d; 5 = parturition in 1–2 d; and 6 = parturition in 0–1 d. Parturition was considered imminent in stage-6 prolarvae that had depleted yolk reserves but had a well-developed oil globule. After staging and measuring the prolarvae, the otoliths were removed, affixed to glass slides, and the otolith radius measured (μm). No more than five prolarvae were examined from any single gestating female.

Otoliths of gestating and planktonic larvae (n=60) were examined with a scanning electron microscope (SEM). To prepare otoliths for viewing, whole larval specimens were dehydrated in successive rinses of 95 and 100% ethanol (24 hours in each) and immersed in Spurr's embedding medium. Notochord length (NL) was measured with the aid of a binocular microscope and calibrated ocular micrometer scale. Each fish was covered with the medium and oriented on its side in a silicone rubber embedding mold (Haake et al., 1982). Curing required 24 hours at 70°C. The otoliths from these larvae were hand ground with 9-μm aluminum oxide microfinishing film and mechanical lapping wheels to produce a midsagittal section from one of the two sagittae. The grinding progress was monitored by frequent inspection of the sample with both transmitted and incident illumination in a metallographic microscope. Final polishing was achieved with 3- and 1-μm diamond pastes. After the final polish, the mounts were rinsed with distilled water and air dried. Immersion and gentle agitation in a 5% solution of ethylenediaminetetraacetic acid (EDTA) (pH adjusted to 7 with potassium hydroxide [KOH]) etched the otoliths for observation under a SEM. Required etching times of 10–40 seconds were determined by periodic rinses and inspection with a light microscope. Owing to shifting of the otoliths in the otic capsules or to twisting or distortion of the larvae, approximately one third of the samples resulted in a somewhat oblique to transverse section through the sagitta. Measurements of radii or increment widths were not significantly affected by this occurrence because otoliths have almost perfect radial symmetry during the earliest postextrusion stages. The etched preparations were then vacuum sputter-coated with gold palladium before viewing with a back-scattered electron detector (BEI) in the SEM. At least two micrographs were taken for each sample, usually at 2,000–3,500× and 10,000×. The photographic images were digitized and measurements were made with the aid of an image analysis software package (Optimus Corp.). A custom program was used for semi-automated measurements of maximum otolith radius (from the center of the otolith primordium) and maximum radius to the presumptive extrusion check. The first 7–10 postextrusion increments were then marked and measured.

Results

The distribution of *S. jordani* larvae in the survey region was quite variable, with catch rates ranging from 0 to 10,670 larvae·10 m^{-2} of sea surface. Like many ichthyoplankton surveys, the distribution of catch rates was strongly skewed. For example, the five largest tows accounted for 3.33% of the effort, yet produced 65.4% of the catch. The overall mean catch rate of larvae was 328 larvae·10 m^{-2} (n=150, $\hat{\sigma}$=1,285, CV=392%).

A total of 2,203 larvae was subsampled from the total catch and aged by optical microscopy. Larvae ranged in age from 0–34 d (median=4 d). Among the aged larvae, the average radius of the otolith measured to the extrusion check (R_0) was 16.88 μm ($\hat{\sigma}$=1.41 μm), with 90% of all values lying in the range 14.5–19.1 μm (Fig. 2).

[1] Eldridge, M. 1994. Tiburon Laboratory, Southwest Fisheries Science Center, NOAA, 3150 Paradise Drive, Tiburon, CA 94920. Unpubl. data.

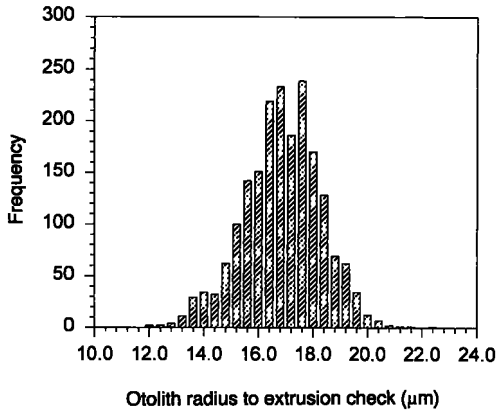

Figure 2

Frequency distribution of the otolith radius, measured to the extrusion check, among aged planktonic larvae, *Sebastes jordani*.

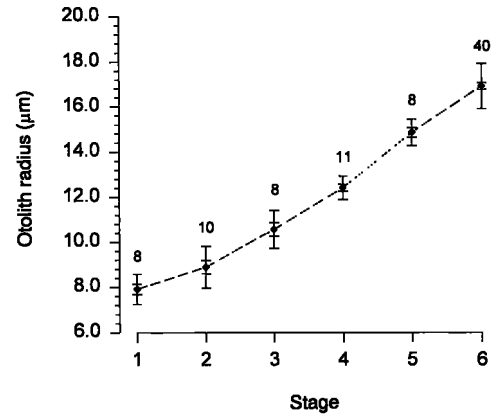

Figure 3

Growth in otolith size with development in gestating prolarvae, *Sebastes jordani*. Means are bracketed by ±1 standard error and ±1 standard deviation (samples sizes above).

A sample of 85 gestating prolarvae were staged from mature female specimens and the total radius of the otolith measured. Results show that prolarval otoliths grow in size as embryonic development proceeds (Fig. 3), and that by attainment of stage 6 the average total otolith radius was 16.91 μm ($\hat{\sigma}$ =1.01 μm). A pooled variance *t*-test (Snedecor and Cochran, 1967), comparing the mean otolith radius at the extrusion check (R_0) of planktonic larvae with the mean total otolith radius of stage 6 gestating prolarvae, was not significant (t=–0.136; df=2,241; P>0.50). This test was powerful, with a difference in the means as small as 0.43 μm being significant at $\alpha = 0.05$. Moreover, an extrusion check was not observed in any otoliths from gestating larvae. These results indicate that the check mark forms at parturition.

Observations of gestating and planktonic *S. jordani* otoliths with the SEM revealed a well-defined microstructure (Fig. 4). The central area comprised a primordial region which often displayed a cluster of etchant-resistant, crystal-like particles (Fig. 4, B and C). Surrounding the particles was a more deeply etched zone presumed to be predominated by matrix material. Distal to this was a calcified region which generally displayed up to three weakly expressed concentric zones. Incremental growth was considered to be very ambiguous here; the core area included this calcified region around the primordium and was defined by a distinct deeply etched zone (Fig. 4, B, C, and D). Distal to the core boundary, regular incremental growth was observable in most of the preparations. Increments in this preextrusion growth phase were typically 0.3–0.6 μm wide but were of low topographical and compositional contrast in the BEI images (Fig. 4, B and C). There were moderately consistent patterns of broader light and dark areas within the region of preextrusion otolith deposition.

The defining boundary for the preextrusion otolith is a deeply etched zone which corresponds to the extrusion check noted in light microscope observations (Fig. 4, A, C, and D). Distal to this landmark, incremental growth consisted of a regular depositional pattern with distinct zonation and gradually increasing spacing (Fig. 4, C–F). There was individual variation in the spacing pattern of the first 5–10 increments. Some larvae had closely packed increments (Fig. 4F), whereas at the other extreme, some larvae exhibited obviously faster otolith growth immediately after extrusion (Fig. 4E).

A comparison of increment widths derived from optical microscopy with those derived from SEM observations shows that otolith microstructure was adequately resolved with the optical system (Fig. 5). Importantly, the SEM did not detect the existence of increments too small to resolve with optical microscopy. Increment widths typically increase in size during the earliest stages of larval growth (e.g. Campana et al., 1987) and this increase was observed in *S. jordani*. The width of the first postparturition increment was approximately 0.6–1.0 μm, which can be resolved by optical methods. Subsequent increments, at least up to day five, slowly increased in size.

A growth model for the first month of life was derived from the age and length data. Length was first log-transformed because NL variance was propor-

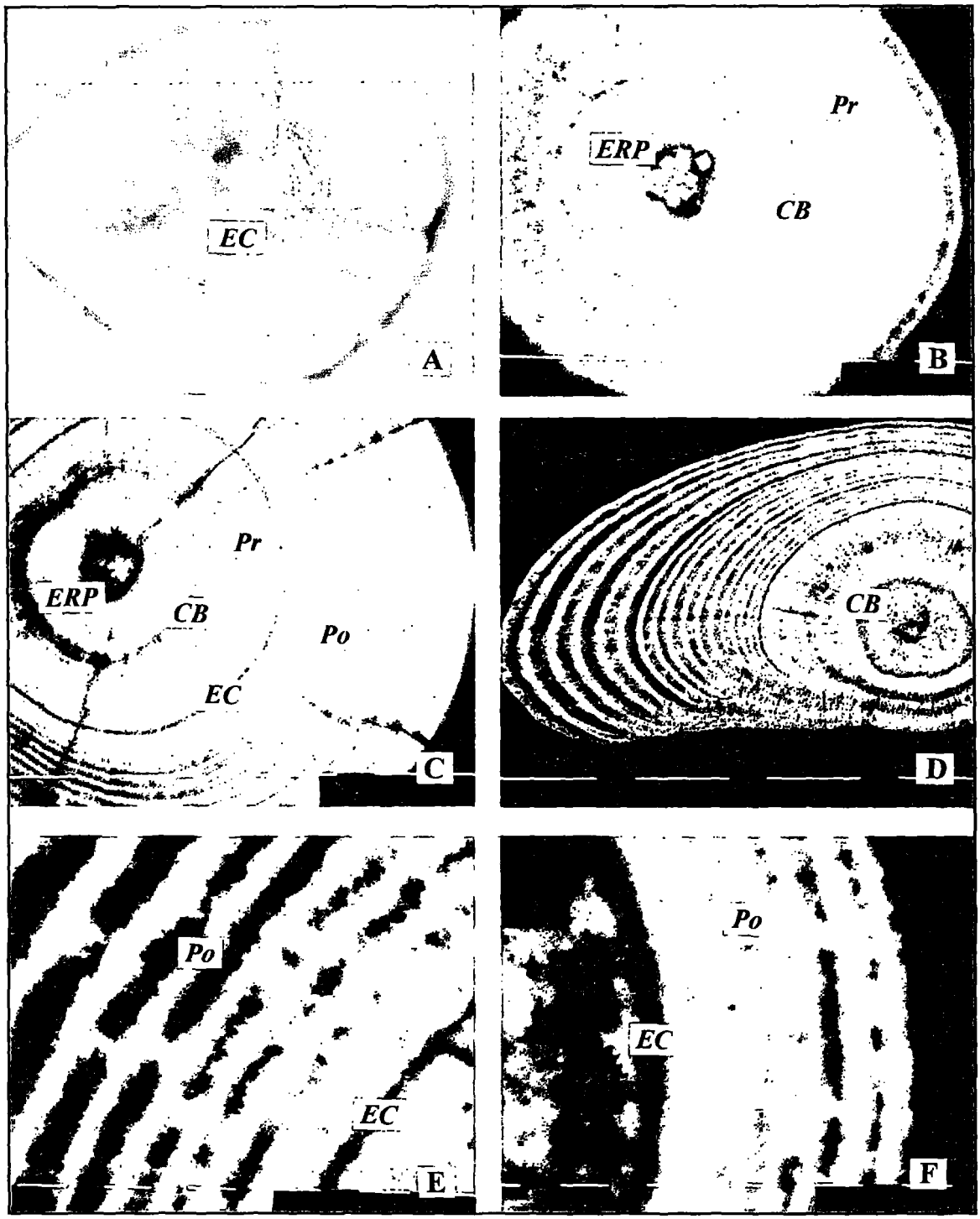

Figure 4

Micrographs of larval shortbelly rockfish, *Sebastes jordani*, otoliths: *EC* = extrusion check; *ERP* = etchant-resistant particles in primordium; *CB* = core boundary; *Pr* = preextrusion incremental growth; *Po* = postextrusion increments. (**A**) optical micrograph (400×) of a wholemount sagitta from a 8.5-mm planktonic larva showing increment microstructure; (**B**) SEM micrograph from a stage–6 gestating prolarva (scale bar = 10 μm); (**C**) sagittal section SEM micrograph from a 7.3-mm planktonic larva (scale bar = 10 μm); (**D**) transverse section SEM from an 8.2-mm planktonic larva (scale bar = 10 μm); (**E**) wide post-extrusion increments (scale bar = 1 μm); (**F**) narrow postextrusion increments (scale bar=1 μm).

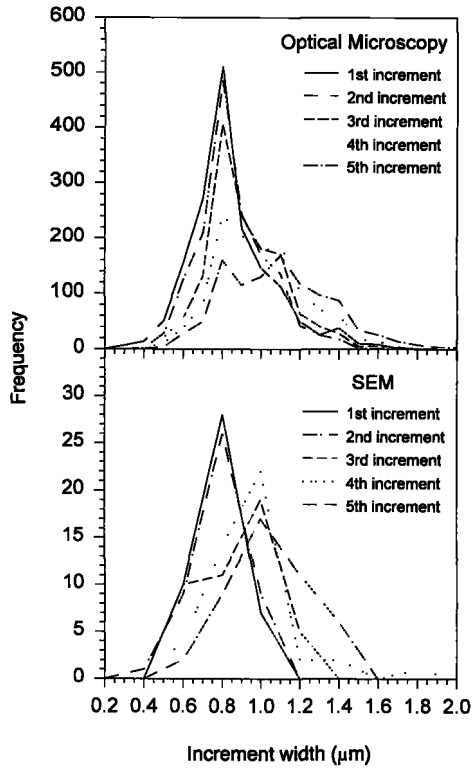

Figure 5

Frequency distributions of the widths of the first five daily increments ($W_1 - W_5$) measured with optical microscopy and SEM.

tional to the mean. A linear ($Y=a + (bx)$) least-squares regression of $\log_e$(NL) on age yielded estimates of b = 0.0272 (s_b=0.000250) and a = 1.640 (s_a=0.00231) with an r^2 value of 0.849 and $\hat{\sigma}^2_{mse}$ = 0.00598. A back transformation of predicted length at age, with bias correction (Miller, 1984), is presented in Figure 6. Also shown in the figure are the original untransformed data and the predicted length-at-age relationship reported by Laidig et al. (1991).

When different readers examined the same otoliths there was good agreement among age estimates (Fig. 7). For example, among the combined total of 87 cross-validations, the three age readers agreed to the day 41% of the time. Moreover, they were within ±1 d 84% of the time and ±2 d 93% of the time. In addition, all paired t-tests for systematic age differences among the readers were not significant (Fig. 7). The statistical power of these tests was such that mean differences in age in the range 0.38–0.88 d were detectable at the α = 0.05 level of significance.

Good agreement among age estimates was also reflected in a high level of certainty in the data. Confidence codes for 67% of all age readings indicated high confidence or better (codes ≥4), 97% were at least moderate confidence (codes ≥3), and only one sample was discarded as unreadable (code=1). Moreover, among the three age readers, the distributions of codes were alike, indicating similar perceptions with respect to the overall clarity of otolith microstructure. There were some differences, however, in the confidence codes of very recently spawned fish and those of older larvae. Fish 1–3 d old were deemed the most difficult to age, whereas ages assigned to older fish (≥7 d) were viewed most confidently.

Discussion

We have shown that a check mark forms in the otoliths of *S. jordani* larvae at parturition. This extrusion check can be identified by its optical characteristics and by its location in the otolith (≈17 μm radius). Penney and Evans (1985) first described an extrusion check in *Sebastes* on the basis of observations of larval redfish in the northwest Atlantic Ocean. These authors described it as "a heavy ring composed of a wide, translucent band followed by a prominent, high-contrast dark band." The check was absent in gestating larvae but was consistently observed in planktonic larvae. Our results are in agreement with theirs and confirm the findings of Laidig

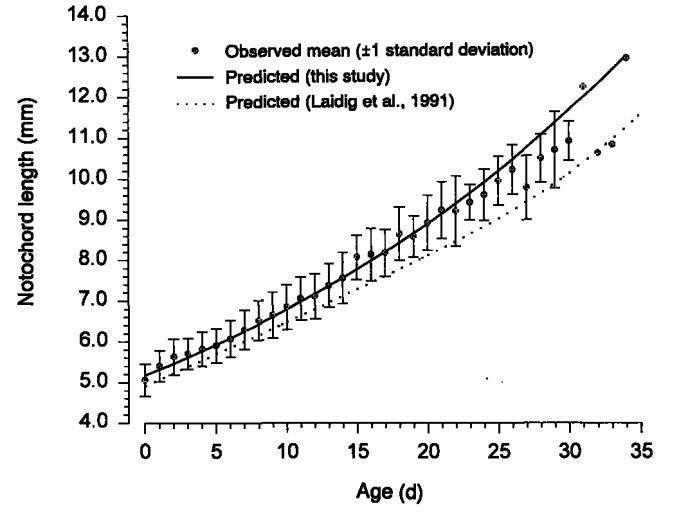

Figure 6

Observed and predicted growth of ethanol-preserved larval shortbelly rockfish, *Sebastes jordani* (means are bracketed by ±1 standard deviation).

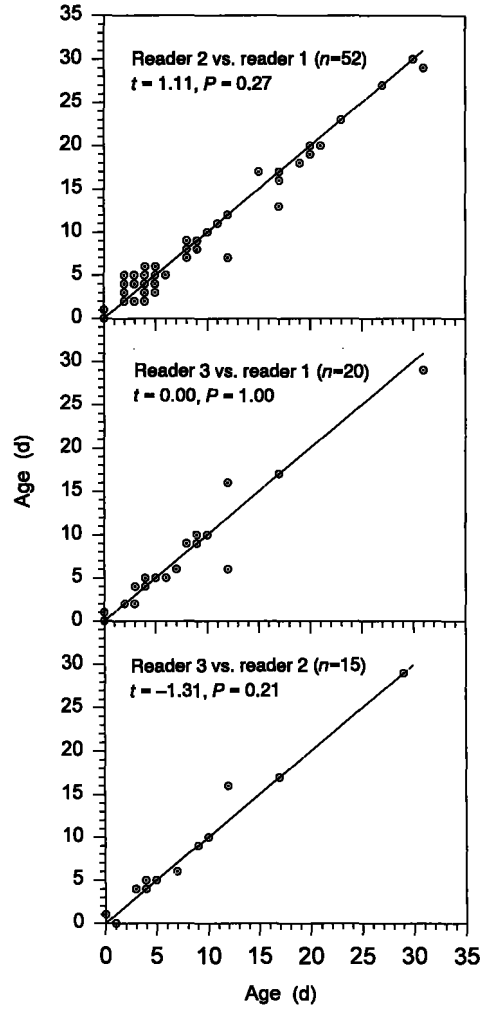

Figure 7

Comparison of age readings of larval *Sebastes jordani* among three different age readers. Diagonal lines represent lines of equality (some points superimposed).

et al. (1991), who first described an extrusion check in the otoliths of *S. jordani*. A distinct "birth mark" has also been observed to form in the otoliths of the viviparous surfperch *Micrometrus minimus* at the time of parturition (Schultz, 1990).

In certain situations otolith size at hatch is known to depend on temperature (e.g. Campana, 1989) and in our study area temperature varies substantially among years (Ralston, 1995; Ralston and Howard, 1995). Because the planktonic larvae we used were collected in 1991, whereas the gestating larvae were collected in 1993, it is important to consider the effect this might have on otolith size at extrusion and the conclusions we have drawn. Results presented in Laidig and Ralston (1995) bear specifically on this issue; i.e. they found no interannual differences in otolith radius at the extrusion check for six species of *Sebastes* (including *S. jordani*) that were sampled over a six-year period (1984–89). However, interannual differences in the width of the first postextrusion increment were observed.

There are, moreover, interspecific differences in extrusion check radius, and these can aid in identifying rockfish species (Laidig and Ralston, 1995). In this regard, *S. jordani* has a particularly large preextrusion radius and can be distinguished 68% of the time on this basis alone. We speculate that the *Sebastes* extrusion check may owe its origin to an osmotic or ionic shock that occurs to the larvae when they first contact seawater and that temporarily alters calcium metabolism.

It is noteworthy that many otoliths exhibited incremental growth during gestation (see also Schultz, 1990). We observed these preextrusion increments in both gestating and planktonic larvae. Although the expression of these structures was quite variable, their existence highlights the importance of accurately locating the extrusion check. We speculate that the physiological basis of preextrusion increments in these larvae is linked to the transmittal of the diel maternal calcium cycle into the ovarian embryonic environment (Mugiya et al., 1981).

The existence of a clear, well-defined mark that is formed at parturition satisfies an important requirement for accurate larval age estimates. An unambiguous starting point for counting increments is a necessary, but not sufficient, condition for obtaining reliable data. Increment counts must also be precise. For *S. jordani* this requirement was met, given the high percent agreement among age readers (93% within ±2 d) and the lack of systematic reader differences in ageing. High precision was also reflected in the relatively high confidence that readers assigned to their work, stemming from the consistent clarity of increment microstructure in the otoliths. Fish 1–3 d old were the most difficult to interpret. This was probably due 1) to optical interference from light diffraction around the otolith margin, 2) to the fact that daily increments present at those ages were the narrowest in width, and 3) to the fact that fewer increments hampered visual recognition of a growth pattern.

A serious potential source of bias in ageing larval fish is the limited resolution of optical microscopes (Neilson, 1992). Studies have shown that optical systems are sometimes incapable of resolving fine increment microstructure (Campana et al., 1987; Jones and Brothers, 1987; Morales-Nin and Ralston, 1990; Secor and Dean, 1992). However, our results for *S. jordani* show that measurements of increment width

obtained with a high-quality optical microscope system produced results similar to an SEM.

From these findings we can conclude that optical increment counts are unbiased with respect to SEM. It need not follow, however, that age estimates are unbiased. If the physiological shock that produces the extrusion check is severe enough, it is possible that increment deposition could be completely arrested for a period of time (Campana and Neilson, 1985). Increment microstructure might then be accurately resolved, but an exact 1:1 correspondence between increments and days may be briefly violated (Geffen, 1992; Neilson, 1992). We have no means of dismissing this possibility from the data we have gathered, although all our results indicate that age estimates of larval *S. jordani* are quite accurate.

Growth of *S. jordani* larvae over the first month of life was well described by a simple exponential model. The predicted length of larvae at parturition (l_0) is 5.17 mm NL, which is quite similar to results presented in Laidig et al. (1991), who reported $l_0 = 4.9$ mm NL. Growth in length then proceeds at about 2.75%·d^{-1}, although there is some indication that growth rate begins to slow sometime around 25 d, which is consistent with the growth stanza model of Laidig et al. (1991). Our observed and predicted lengths at age are ~5–15% higher than the data presented in that study. This discrepancy is possibly due to interannual differences in larval growth, because their samples were collected in 1989 and ours were from 1991. Woodbury and Ralston (1991) reported comparable interannual differences in the growth of five species of pelagic juvenile rockfish, including *S. jordani*.

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